Proliferative Assays for T Cell Function

A number of agents can specifically or nonspecifically induce T cell activation, resulting in cytokine production, cytokine receptor expression, and ultimately proliferation of the activated T cells. Although proliferation is not a specific effector function of T lymphocytes—in contrast to helper function for B lymphocytes (*UNIT 3.10*) or cytotoxicity (*UNIT 3.11*)—proliferation assays are reliable, simple, and easy to perform and have been widely used to assess the overall immunocompetence of an animal. In addition, the assays described in this unit form the basis for identifying the appropriate cellular population that might be used to obtain T cell clones (*UNIT 3.13*) or T cell hybridomas (*UNIT 3.14*).

The assays have been divided into two groups on the basis of whether they are used to stimulate primed or unprimed T lymphocytes. Basic Protocol 1 describes the use of agents that are capable of activating unprimed T lymphocytes in culture either by pharmacologic means (calcium ionophore and phorbol ester stimulation), by direct cross-linking of the T cell receptor (TCR) on a large percentage of responder cells (anti-CD3, anti-TCR-γδ, or anti-TCR-αβ monoclonal antibodies), by cross-linking the receptors on certain subpopulations of T cells with monoclonal antibodies specific for the *V* regions of β chains of the TCR (anti-Vβ) or with enterotoxins specific for certain *V*β-chain regions, or by indirectly cross-linking the TCR (lectins or monoclonal antibodies to non-TCR antigens). Alternate Protocol 1 describes the use of plate-bound antibodies specific for the TCR to stimulate proliferation. Alternate Protocol 2 describes the activation of unprimed T cells to cell-associated antigens in the mixed leukocyte reaction (MLR). Support Protocol 1 describes the preparation and use of T cell–depleted accessory or stimulator cells and Support Protocol 2 describes methods for blocking accessory cell proliferation. Finally, Basic Protocol 2 describes the induction of a T cell proliferative response to soluble protein antigens or to cell-associated antigens against which the animal has been primed in vivo.

$CD4^+CD25^+$ T cells are a naturally occurring population of cells that suppress immune responses to auto- and allo-antigens, tumor antigens, and infectious agents in vivo. In vitro, $CD4^+CD25^+$ cells suppress the proliferative responses of $CD4^+$ and $CD8^+$ T cells. In contrast to conventional T cells, $CD4^+CD25^+$ cells do not proliferate to TCR stimulation, but do proliferate in response to TCR stimulation in the presence of IL-2. Basic Protocol 3 describes the methods used to evaluate the non-responsive state of $CD4^+CD25^+$ T cells as well as the methods used to determine and quantitate their suppressive capacity. Alternate Protocol 3 describes the procedure needed for short term expansion of $CD4^+CD25^+$ T cells and the method used to assay the suppressive function of the pre-activated cells.

The assays in this unit employ murine T lymphocytes. Induction of proliferative responses of murine B lymphocytes is described in *UNIT 3.10*. Related assays for use with human peripheral blood lymphocytes are described in *UNIT 7.9*.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

ACTIVATION OF UNPRIMED T CELLS

BASIC PROTOCOL 1

Unprimed T cells can be induced to proliferate by a variety of agents, including pharmacological agents, anti-CD3/TCR or anti-Thy-1 monoclonal antibodies, enterotoxins and lectins. The Commentary briefly describes the specificities of these agents, while Table 3.12.1 lists sources and concentrations for use in this protocol. Although this procedure is intended to measure proliferation of T cells specifically, in many cases induction of T

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Table 3.12.1 Agents Used to Activate Unprimed T Cells in Proliferative Assays

Agent[a]	Source[b]	Concentration	Accessory cells[c]	Mode of action
PMA	SIG	1-10 ng/ml	No	Use with ionomycin or A23187; pharmacologic
Ionomycin	CAL	200-500 ng/ml	No	Use with PMA; pharmacologic
A23187	CAL	100-500 ng/ml	No	Use with PMA; pharmacologic
PHA	WD	1-5 μg/ml	Yes	Indirect TCR cross-linking
Con A	PH	1-10 μg/ml	Yes	Indirect TCR cross-linking
Anti-Thy-1	PG mAb-G7	1-50 μg/ml	Yes[c]	Indirect TCR cross-linking
Anti-CD3	PG HM-CD3	0.1-5 μg/ml	Yes[c]	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-αβ	PG HM-AB-TCR	0.1-10 μg/ml	Yes[c]	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-γδ	PG HM-GD-TCR-1; HM-GD-TCR-3	0.1-100 μg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-8.1, 8.2[c]	PG MM-Vβ-TCR-1	0.1-100 μg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-6[c]	PG RM-Vβ-TCR-2	0.1-100 μg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-11	PG RM-Vβ-TCR-3	0.1-100 μg/ml	No	Use plate-bound; direct TCR cross-linking
Staph tox A	TT	1-10 μg/ml	Yes[c]	Vβ-1,3,10,11,17-receptor specificity
Staph tox B	TT; SIG	1-100 μg/ml	Yes[c]	Vβ-3,7,8,17-receptor specificity
Staph tox E	TT	1-10 μg/ml	Yes[c]	Vβ-11,15,17-receptor specificity

[a]Abbreviations: PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; Con A, concanavalin A; Staph tox A, B, & E, *Staphylococcus* enterotoxins A, B, & E.

[b]Supplier addresses and phone numbers are provided in *APPENDIX 5*. Abbreviations: CAL, Calbiochem; PG, Pharmingen; PH, Pharmacia LKB; SIG, Sigma; TT, Toxin Technology; WD, Wellcome Diagnostics.

[c]When using anti-CD3 and anti-TCR antibodies in soluble form (rather than plate-bound), accessory cells are required. When using Staph enterotoxins, accessory cells must express appropriate MHC class II molecules. Accessory cell dependence is not absolute with anti-Thy-1 antibodies.

cell proliferation is dependent on the presence of non–T cells that function as accessory cells. The latter provide additional costimulatory signals for T cell proliferation as well as cross-link (via their Fc receptors) monoclonal antibodies bound to cell-surface antigens. The requirement for non–T accessory cells varies with the nature of the stimulatory ligand and can range from absolute dependence to accessory cell–independent T cell activation (see Table 3.12.1). The activation is calculated after determining the difference in incorporation of [^{3}H]thymidine between stimulated and control cells.

Materials

Complete RPMI-5 and RPMI-10 media (*APPENDIX 2A*)
Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (*UNIT 3.1*)
Activating agent(s) (Table 3.12.1)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Accessory cells: unfractionated mouse spleen cell suspension, irradiated or treated with mitomycin C (see Support Protocol 2) or T cell–depleted (see Support Protocol 1)
[^{3}H]thymidine (*APPENDIX 3D*)

15- and 4-ml disposable, polystyrene conical tubes with screw caps
Low-speed centrifuge with Sorvall H-1000B rotor (or equivalent)
1-, 5-, and 10-ml disposable polystyrene pipets
96-well flat- or round-bottom microtiter plates with lids (Costar)
25- to 100-µl single- and multichannel pipettors with disposable tips

Additional reagents and equipment for removing organs (*UNIT 1.9*), preparing single-cell suspensions (*UNIT 3.1*), and counting, labeling, and harvesting cells (*APPENDIX 3*)

1. Prepare responder leukocyte suspensions from thymus, spleen, or lymph node in complete RPMI-5 as described in *UNIT 3.1*.

   The size of the intended experiment dictates the number of organs to be collected. See annotation to step 3 for an indication of cell number required, and UNIT 3.1 for number of cells per organ. Spleen, thymus, and lymph node can be used as responder cells, while only spleen is a source of accessory cells. Purified T cells or subpopulations of T cells (i.e., CD4$^+$ or CD8$^+$) cells may also be used. See UNITS 3.1-3.6 for enrichment/depletion methods.

2. Centrifuge single-cell suspensions in 15-ml conical tubes for 10 min in Sorvall H-1000B rotor at ~1000 rpm (200 × *g*), room temperature, and discard supernatant.

3. Resuspend cell pellet in complete RPMI-5. Count responder cells and adjust to ~10^6 cells/ml with complete RPMI-10.

   While this concentration (1×10^6 cells/ml or 2×10^5 cells/well) will give satisfactory responses with most cell populations, it is useful to compare 2, 4, and 8×10^5 cells per well in initial pilot experiments. If unfractionated spleen or lymph node cells are used as the responder population, sufficient accessory cells are present and there is no need to supplement the cultures with additional cells. However, if highly purified T cells or T cell subpopulations are used as responders, it will be necessary to add non–T accessory cells depending on the nature of the activating agent (see Table 3.12.1). This is most easily accomplished by adding increasing numbers ($0.1, 0.5$, and 1.0×10^5) of syngeneic spleen (accessory) cells in 0.1 ml to 2×10^5 T cells in 0.1 ml (see Support Protocol 1). Also, a meaningful comparison of the responsiveness of different cell populations requires titrations of both the activating agents as well as the responding cell populations, and a kinetic experiment.

4. Prepare working solutions of activating agents in 4-ml conical tubes at room temperature as follows. For MAb, toxin, or lectin, make a series of four dilutions from

1 mg/ml stock solutions—e.g., 100, 30, 10, and 3 μg/ml in PBS. For the pharmacological agent, make single dilutions of 100 ng/ml solution of PMA and 1 μg/ml A23187 (or 4 μg/ml ionomycin) in PBS.

If MAb in supernatant or ascites form is being used, at least four dilutions should also be used. Working solutions should be used immediately, since the various proteins, especially MAb, may bind to the plastic.

See Table 3.12.1 for Vβ specificities of staphylococcal enterotoxins. It is essential to verify that the mouse strain employed expresses the MHC class II surface molecules for which the enterotoxin has a specific binding affinity. See Marrack and Kappler (1989) for further discussion of various enterotoxins and their specificities.

5. Add 20 μl of each dilution of activating reagent (MAb, enterotoxin or lectin) to each of three wells of a 96-well flat- or round-bottom microtiter plate. Include control wells with 20 μl of PBS only. Add 20 μl PMA or calcium ionophore at the single concentration indicated in step 4, as the dose-response curve for these agents is extremely narrow.

A series of four dilutions will form one row of each microtiter plate, allowing for efficient organization of the plates.

6. To the wells of the 96-well microtiter plate containing activating agent, add 2×10^5 cells in 0.2 ml.

7. Place microtiter plates in a humidified 37°C, 5% CO_2 incubator for 2 to 4 days.

Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions and must be determined empirically (see Critical Parameters and Troubleshooting).

8. Add [^{3}H]thymidine to each well. Return the plates to CO_2 incubator to pulse 18 to 24 hr. Harvest cells using a semiautomated sample harvester and measure cpm in β scintillation counter.

9a. Compute the data as the difference in cpm of stimulated (experimental) and control (no activating agent added) cultures. This is done by subtracting the arithmetic mean of cpm from triplicate control cultures from the arithmetic mean of cpm from corresponding stimulated cultures. The results are referred to as "Δ cpm."

9b. Alternatively, compute the data as the ratio of cpm of stimulated and control cultures. This is done by dividing the arithmetic mean of cpm from stimulated cultures by the arithmetic mean of cpm from control cultures. The results are referred to "SI" (stimulation index).

The second method (step 9b) has the disadvantage that small changes in background values will result in large changes in SI and should be interpreted with caution. In most publications, Δ cpm rather than SI values are preferred.

ALTERNATE PROTOCOL 1

ACTIVATION OF UNPRIMED T CELLS WITH PLATE-BOUND ANTIBODIES

Although it is possible to induce T cell activation with monoclonal antibodies to the CD3/TCR complex in solution during culture, such activation depends on cross-linking of the antibody by Fc receptor–bearing accessory cells. This protocol describes the use of monoclonal antibodies to the CD3/TCR complex by coupling them to the wells of the microtiter plates. The T cell proliferative response induced under these conditions does not require the presence of significant numbers of accessory cells, although the responses obtained may be suboptimal (Jenkins et al., 1990).

Use of this protocol is recommended for use with those antibodies to the CD3/TCR complex which bind poorly to the Fc receptor present on murine accessory cells and which do not induce T cell activation in soluble form. Although all monoclonal antibodies readily couple to plastic under these conditions, it is very difficult to induce a proliferative response with certain antibodies such as the G7, anti-Thy-1 monoclonal antibody. In such cases, the conditions described in Basic Protocol 1 should be followed.

Additional Materials

PBS (*APPENDIX 2A*), room temperature and 4°C
1 mg/ml purified anti-CD3 or anti-TCR MAb in PBS (for nonspecific activation of T cells) *or* 1 mg/ml purified anti-Vβ or anti-TCR-γδ MAb in PBS (for activation of T cells with specific receptors; see Table 3.12.1)

1. In 4-ml conical polystyrene tubes, prepare a series of four dilutions of MAb from sterile 1 mg/ml stock solutions—e.g., 100, 10, 1, and 0.1 μg/ml—using room temperature PBS.

   Sources and recommended concentrations of monoclonal antibodies can be found in Table 3.12.1; since MAb will bind to plastic, the working dilutions should be used immediately.

   The ability of anti-TCR antibodies to cross-link receptor molecules varies depending on the purity of the MAb preparation and the affinity of the MAb for the TCR/CD3 complex. Optimum dilutions will have to be determined in dose-response experiments. Alternatively, preparations of ascites fluid from the MAb can be tested at different dilutions (e.g., 1:100, 1:200, 1:400, and 1:800), but use of purified antibody will allow for better standardization of the assay.

   Because the efficacy of MAb-induced activation depends on the amount of antibody bound to the bottom of the wells, it is crucial to make the dilutions in a buffer without any additional source of proteins such as FBS or albumin; these would compete with the binding of the antibody, and therefore reduce the responsiveness. For this reason, it is also not recommended to perform the assay with culture supernatants of the appropriate hybridomas.

2. Add 30 μl of each concentration of MAb solution to each of three wells of a 96-well round-bottom microtiter plate. Include control wells of 30 μl PBS only.

   A series of four dilutions will form one row of each plate, allowing for efficient organization of the plates. Consistently better responses are seen with round-bottom (compared with flat-bottom) plates in antibody-mediated experiments.

   Most often, optimal responses are seen with 10 μg/ml antibody. There is no point in adding more than the indicated amount of antibody, since the maximum amount that can bind to surface of the wells is ~2 to 3 μg (A.M.K., unpub. observ.).

3. Cover the plate and gently tap its side to ensure complete covering of the bottom of the wells. Incubate plates 90 min at 37°C. During incubation, proceed to step 4.

   During this incubation, the antibodies bind to the plastic in the wells for subsequent cross-linking of the T cell receptors on responding T cells. Plates can also be prepared the night before an experiment and kept in the refrigerator overnight, after the 37°C incubation.

4. Prepare responder cell suspensions as in Basic Protocol 1, steps 1 to 3.

   Highly purified T cell populations can be used in these studies as the proliferative response induced is accessory cell–independent. However, the presence of non–T accessory cells does not interfere with the proliferative response.

5. Wash the wells of the incubated plates by adding 200 μl cold PBS and inverting the plates with a flick of the hand on a stack of paper towels placed in a tissue culture hood. Repeat washing procedure two more times to remove excess antibody.

6. To the wells of the washed plates, add ~2×10^5 cells in 0.2 ml.

If cells are not ready at this stage, plates may be kept in the refrigerator overnight after 100 µl PBS has been added. Presumably, longer storage periods should be acceptable, but our experience is limited to ≤4 day periods. The PBS should be removed before the cells are added.

Most cell populations will give peak responsiveness at this cell dosage, but pilot experiments should be performed to establish optimal conditions.

7. Proceed as in steps 7 to 9 of Basic Protocol 1, but incubate cultures for 2 to 3 days before adding [^{3}H]thymidine.

Kinetic assays should be performed to determine the optimum culture period.

ALTERNATE PROTOCOL 2

T CELL PROLIFERATION IN MIXED LYMPHOCYTE CULTURES

In the mixed lymphocyte culture (MLC) or reaction (MLR), suspensions of responder T cells are cultured with allogeneic stimulator lymphocytes. The activating stimulus is the foreign histocompatibility antigen (usually MHC class I or class II molecules) expressed on the allogeneic stimulator cells. Responder cells need not be primed because a sufficiently high number of T cells in the MLC will respond to the stimulator population. If the stimulator cell population contains T cells, their uptake of [^{3}H]thymidine must be prevented by irradiation or treatment with mitomycin C; alternatively the stimulator cell suspension can be depleted of T cells (see Support Protocols 1 and 2).

Additional Materials (also see Basic Protocol 1)

Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (*UNITS 1.9 & 3.1*) *or* purified T cells or T cell subpopulations (*UNITS 3.1-3.6*)

Stimulator cells: allogeneic mouse spleen cells that differ from the responder cells at *H-2* or *Mls* loci, irradiated or treated with mitomycin C (see Support Protocol 2) or T cell–depleted (see Support Protocol 1)

1. Prepare responder cell populations as in Basic Protocol 1, steps 1 to 3. Although unfractionated cell populations can be used as responders in certain situations, it may be preferable to use purified T cells or T cell subsets.

To estimate the MLR of a cell population, it is necessary to perform a dose-response assay with different numbers of responder cells. Typically, three replicate wells are set up containing each of the following: 0.5, 1, 2, and 4×10^5 cells (optimal responses are usually obtained with the latter two densities). The setup for these four cell densities will occupy one row (12 wells) of a microtiter plate.

For thymocytes, it may be necessary to use 8×10^5 cells per well because the frequency of responding T cells is lower; the lowest number of responder cells could then be 1×10^5 and the doses in between would be 2 and 4×10^5. Using this range of higher numbers of responder cells may also be preferred when experimental manipulations are expected to reduce the frequency of responding T cells.

2. To a 96-well microtiter plate, add 5×10^4 to 4×10^5 responder cells in 0.1 ml to each well. For each experimental group, set up three replicate wells.

Stimulation of leukocytes for proliferation in 96-well microtiter plates can be run in parallel with cytotoxic T lymphocyte (CTL) generation (UNIT 3.11), which is performed in 24-well microtiter plates. For example, cells can be diluted to 4×10^6 cells/ml and added to 24-well plates in 1.0 ml/well for CTL generation and to 96-well plates in 0.1 ml/well for proliferation.

3. Prepare a single-cell suspension of irradiated or mitomycin C–treated stimulator cells. Alternatively, prepare a suspension of T-cell depleted stimulator cells. Add 0.1 ml to each well of the plates containing responder cells.

The optimum number of stimulator cells must be determined for each MLC and for different responder cells. For a range of responder cells from 0.5-4 × 10^5, test stimulator cells at densities of 2, 4, and 8 × 10^6/ml (i.e., 2, 4, and 8 × 10^5/well). It should be noted that the stimulator cell suspension provides both the specific antigen to be recognized by the responder T cells as well as nonspecific accessory cells. If highly purified T cells are used as the responder population, it is therefore not necessary to supplement the cultures with non–T accessory cells syngeneic to the responder T cells.

Separate wells with control cultures should be set up that include—for each dose of responder and stimulator cells—replicate wells of responder cells with irradiated or mitomycin C–treated syngeneic stimulator cells. Values obtained from these controls reflect "background" proliferation values (see Basic Protocol 1, step 9). Other negative controls often included are wells with stimulator cells alone and wells with responder cells alone. These are not used for the calculation of the data, but are useful to compare with the background proliferation values; the latter should not be much higher (<2-fold) than those obtained with stimulator or responder cells alone. Higher background values indicate potential autoreactivity.

4. Follow Basic Protocol 1, steps 7 to 9, but incubate the cultures for 3 to 6 days.

Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions, and must be determined empirically (see Critical Parameters and Troubleshooting).

DEPLETION OF T CELLS FROM ANTIGEN-PRESENTING/STIMULATOR CELL SUSPENSIONS

SUPPORT PROTOCOL 1

Although normal unfractionated spleen cell populations can be used as a source of accessory cells, in certain types of experiments it may be preferable to use spleen cell populations from which the T cells have been removed. This procedure ensures that none of the observed proliferative responses of the responder population result from T cell factors derived from the accessory cell population. For example, even T cells whose cell division has been blocked (see Support Protocol 2) can produce cytokines. In the following steps, T cell–depleted spleen cell suspensions are prepared using a lytic monoclonal antibody to the T cell antigen, Thy-1. Because almost all the antigen presentation or stimulator cell activity in spleen resides in the non–T cell fraction, this procedure also leads to enrichment of functional antigen-presenting cell function. Further enrichment of antigen-presenting cells (APC) by flotation of the T cell-depleted spleen cells on Percoll gradients is also described. Other procedures leading to enrichment of APC are described elsewhere; the method described in *UNIT 3.7* does not deplete T cells and therefore is not recommended here; the method described in *UNIT 3.15* leads to higher levels of enrichment that are not required in the protocols presented here.

Additional Materials

Spleen cells from nonimmunized mice
Hanks' balanced salt solution (HBSS; *APPENDIX 2A*)
Low-Tox rabbit complement (Cedarlane), reconstituted with ice-cold distilled water and filter-sterilized
Anti-Thy-1.2 ascites (HO-13-4; ATCC no. TIB 99) or anti-Thy-1.1 ascites (HO-22-1; ATCC no. TIB 100; alternatively, see Table 3.4.1 for other anti-Thy-1 MAb and *UNIT 2.6* for production of ascites)
70% Percoll solution (see recipe; *UNIT 3.8*)

1. Centrifuge the spleen cell suspension derived from single spleen down to a pellet.

   The spleen cells should always be from nonprimed animals and should be syngeneic to the responder T cells unless they are to be used as stimulator cells in the MLC.

2. To the pellet, add 0.9 ml HBSS, 0.1 ml complement, and 25 µl anti-Thy-1 ascites.

   If cells from more than a single spleen are needed, the procedure should be scaled up accordingly.

   The choice of anti-Thy-1 reagent to be used depends on the strain of animal from which the spleen was derived. The great majority of commonly available mouse strains (except AKR) express the Thy-1.2 allele.

3. Incubate the mixture at 45 min in a 37°C water bath.

4. Centrifuge 10 min in Sorvall H-1000B rotor at ~1000 rpm (200 × *g*), room temperature, and discard supernatant. Resuspend pellet in HBSS and wash two more times.

5. Count viable cells (*APPENDIX 3*) and resuspend in complete RPMI-10 or PBS for inactivation as in Support Protocol 2, or in HBSS to prepare low-density accessory cells (see below).

   The T cell–depleted spleen cell population is comprised of B cells, macrophages, and dendritic cells. Further enrichment of cells with enhanced accessory cell function can be obtained by fractionation of this population on Percoll.

6. Dilute 70% Percoll solution to 55% by mixing 23.58 ml of the 70% Percoll with 6.42 ml HBSS. Resuspend T cell–depleted spleen cells from step 5 in HBSS at 20×10^6 cells/ml.

7. Layer 3 ml cell suspension over 3 ml of 55% Percoll solution in a 15-ml conical centrifuge tube.

8. Spin 13 min in H-1000B rotor at 3000 rpm (1900 × *g*), room temperature.

9. Remove cells that band at the Percoll/HBSS interface with a 5-in. Pasteur pipet and wash 3 times in HBSS as in step 4.

10. Count viable cells and resuspend in complete RPMI-10 for inactivation according to Support Protocol 2.

    The population obtained from steps 6 to 10 is comprised of large cells including macrophages, dendritic cells, and activated B lymphocytes. This population of cells is enriched in accessory cell function. When used in either of the basic protocols with purified T responder cells, fewer of the Percoll-purified cells should be needed to provide accessory function.

SUPPORT PROTOCOL 2

BLOCKING CELLULAR DIVISION OF ACCESSORY/STIMULATOR CELLS

There are two situations in which inhibition of accessory or stimulator cell division should be blocked. When purified T cells rather than unfractionated lymphoid populations are used in Basic Protocol 1, cultures are frequently supplemented with accessory cells syngeneic to the responder T cells. If accessory cell DNA synthesis is inhibited, one can then be certain that the resultant proliferative response is comprised entirely of responder T cells and does not contain a component of recruited B cell proliferation derived from the accessory cell populations. In the MLR, the stimulator cells are spleen cells from mice that differ from the responder cells in *H-2* and/or *Mls* gene expression (see *APPENDIX 1*, Tables A.1C.1 and A.1F.1) and they can also recognize alloantigens on the responder cells. This responsiveness of stimulator cells against responder cells in an MLR (so-called back-stimulation) must be prevented by blocking cellular division. This can be done by

treatment of stimulator cells with mitomycin C (a DNA cross-linking reagent) or by γ irradiation. Many investigators prefer mitomycin C treatment when antigenic differences encoded for by *Mls* genes are to be measured, or when an irradiation source is not available. For more information on the loci encoding *Mls* genes, see Tables A.1F.2 and A.1F.3.

Mitomycin C Treatment

Additional Materials (also see Basic Protocol 1)

Mitomycin C (Sigma; store in dark)

1. In a 15-ml aluminum foil–wrapped tube, prepare a solution of mitomycin C in PBS at 0.5 mg/ml and filter sterilize.

   Since mitomycin C is very light-sensitive, it is necessary to prepare a fresh stock solution each day for each experiment.

2. Prepare spleen cell suspension as described in Basic Protocol 1, steps 1 and 2, at a concentration of 5×10^7 cells/ml in PBS.
3. Add mitomycin C to a final concentration of 50 μg/ml (100 μl/ml of cell suspension) and wrap the tube in aluminum foil. Incubate 20 min at 37°C.
4. Add an excess of complete RPMI-5 (i.e., fill tube with ~12 ml) and centrifuge 10 min in Sorvall H-1000B rotor at 1200 rpm ($300 \times g$). Discard supernatant and repeat washing procedure two more times.

   Three washes are crucial, because any traces of mitomycin C left among the cells will reduce proliferative responses when the cells are added to an MLC.

5. Resuspend pellet in complete RPMI-10. Count cells with hemacytometer. Adjust to desired concentration as described in the annotation to Basic Protocol 1, step 6.

Irradiation Treatment

Prepare a spleen cell suspension as described in Basic Protocol 1, steps 1 to 3, at a final concentration of 5-10 $\times 10^6$ cells/ml in complete RPMI-10. Using a source of ionizing irradiation (^{60}Co or ^{137}Cs γ-irradiator; e.g., Gammacell 1000, Nordion), deliver 1000 to 2000 rad of irradiation to the cells.

This dose range of irradiation is suitable for most immunologic applications employing spleen cell suspensions. However, antigen presentation by different spleen cells is differentially affected by irradiation (Ashwell et al., 1984): at low doses (500 to 1000 rad), antigen-presenting function of B cells is preserved; after doses of 1100 to 2000 rad, a substantial decline is observed; and doses >2000 rad abolish the participation of B cells as APC. Macrophages and dendritic cells, on the other hand, maintain antigen presentation through doses of 3000 rad. To ensure that B cells do not participate in the responses measured, some investigators prefer to use doses of 2000 rad. However, responsiveness to *Mls* antigens can best be measured with stimulator cells that received doses of <1000 rad, since B cells present *Mls* more effectively. Alternatively, *Mls* responsiveness can be measured after mitomycin C treatment of stimulator cells, since it also preserves the antigen-presentation function of B cells.

When transformed cell lines are used as antigen-presenting or accessory cells, higher doses must be used to ensure blockage of cell division. The appropriate dose will have to be determined empirically for each cell line, but is likely to be at least 5000 rad; some transformed cell lines require as much as 10,000 to 12,000 rad, and may be more sensitive to mitomycin C treatment.

BASIC PROTOCOL 2

BASIC PROTOCOL 2

ACTIVATION OF PRIMED T CELLS

Proliferative responses to viruses, protein antigens, minor transplantation antigens, and the male H-Y antigen require in vivo immunization followed by in vitro stimulation. Furthermore, enhanced proliferative responses to those antigens that will generate primary in vitro responses (i.e., MHC antigens) can be obtained by in vivo priming. Multiple immunizations usually elevate in vitro responses.

To immunize animals for in vitro secondary responses to soluble protein antigens or peptides, dissolve antigens and emulsify in complete Freunds adjuvant (*UNIT 2.5*). For strong responses by draining lymph node cells, immunize animals in a hind footpad. For strong responses by spleen cells, immunize intraperitoneally. Tail-base immunization also can be used as an efficient route of immunization; follow procedure for intradermal injection. To prime animals against cellular antigens, inject intraperitoneally with 1-5 × 10^7 cells that express the antigen. Immunization protocols are described in *UNIT 1.6*.

Within 2 to 3 weeks after in vivo priming, in vitro responsiveness of primed T cells can usually be measured. This assay is often used as a preparation for subsequent in vitro cloning procedures (*UNIT 3.14*) and T cell hybridoma preparation (*UNIT 3.13*).

Materials

Complete RPMI-10 medium (*APPENDIX 2A*)
Responder cells: Purified T cells isolated from lymph nodes (*UNITS 3.1-3.6*) of in vivo primed mice
Antigen: 1 mg/ml sterile protein antigen(s) (*UNIT 3.13*), in PBS *or* suspension of irradiated or mitomycin C–treated stimulator cells expressing alloantigens at 8 × 10^6 cells/ml (*UNIT 3.11*, Support Protocol) in complete RPMI-10 medium (*APPENDIX 2A*)
Accessory cells: suspension of irradiated or mitomycin C–treated (or T cell–depleted) spleen cells syngeneic to the responding T cells at 5 × 10^6 cells/ml in complete RPMI-10 medium

4-ml conical tubes
96-well flat-bottom microtiter plates with lids

1. Follow Basic Protocol 1, steps 1 to 3, for preparation of responder cells.
2. Prepare 4-fold dilution series of the antigens in 4-ml conical tubes, using complete RPMI-10.

   The following dilutions are recommended: 100, 10, 1, and 0.1 μg/ml protein antigens and 8, 4, 2, and 1 × 10^6 cells/ml of stimulator cells in complete medium.

3. Add antigens to 96-well flat-bottom microtiter plates, at 30 μl/well for protein antigens or 100 μl/well for cellular antigens. For each experimental group, set up three replicate wells and include control wells with medium only (no antigen).

   By using four concentrations of antigens and three replicate wells for each dose, one row of a microtiter plate will cover the entire tested range.

4. Add responder T cells in 0.1 ml to each well.

   Purified T cells are recommended; otherwise extremely high background values may be obtained. This appears to be due in part to proliferation of recruited cells (T and non–T) that are not antigen-specific. If unfractionated lymph node cells from recently primed mice are used, add 1-2 × 10^5 cells per well and proceed to step 6.

5. If purified lymph node T cells specific for protein antigens are used, add 0.1 ml of accessory spleen cells syngeneic to the donor of the responder T cells at 5 × 10^5 cells per well.

Purified T cells require an exogenous source of accessory non–T cells. Accessory cells function both as antigen-presenting cells and as a source of undefined "second signals." They are not required for cell preparations primed against cellular antigens, because accessory cell function is provided by the stimulator cells.

6. Proceed as in Basic Protocol 1, steps 7 to 9.

Culture periods before labeling can vary widely and kinetic assays should be performed. In general, for T cells from primed mice, it is likely that the response will peak at day 4 or 5.

ACTIVATION OF CD4$^+$CD25$^+$ T CELLS AND ANALYSIS OF THEIR SUPPRESSIVE FUNCTION

BASIC PROTOCOL 3

Although T cells proliferate in response to a TCR stimulus as described in Basic Protocol 1, a subset of CD4$^+$ T cells, CD4$^+$CD25$^+$ cells, do not proliferate in response to stimulation through their TCR. Moreover, pure CD4$^+$CD25$^+$ cells do not proliferate in response to the combination of TCR stimulation and anti-CD28. The addition of IL-2 in the presence of TCR stimulation, results in proliferation. This protocol describes the use of anti-CD3 as a TCR stimulus in the presence of anti-CD28 or IL-2 to assess the proliferative responses of CD4$^+$CD25$^+$ T cells. Subsequently, CD4$^+$CD25$^+$ cells are co-cultured with CD4$^+$CD25$^-$ cells to assess their suppressive function.

Materials

CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells (*UNIT 3.5A*)
Complete RPMI-10 medium (*APPENDIX 2A*)
Accessory cells: T-cell depleted mouse spleen cell suspension, irradiated (*UNIT 3.5A* or Support Protocol 1)
Purified anti-CD3 (BD PharMingen)
Mouse or human IL-2 (PeproTech or Roche)
Purified anti-CD28 (BD PharMingen)
[^{3}H] thymidine (*APPENDIX 3D*)

96-well flat bottom microtiter plates with lids
37°C, 5% to 7% CO_2 humidified incubator
Semiautomated sample harvester (*APPENDIX 3*)
β scintillation counter

Additional reagents and equipment for removing organs (*UNIT 1.9*), preparing single-cell suspensions (*UNIT 3.1*), and counting, labeling, and harvesting cells (*APPENDIX 3*)

Prepare cells and solutions

1. Prepare CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cell suspensions in RPMI-10 as described in *UNIT 3.5A*. Count cells and adjust CD4$^+$CD25$^-$ cells and CD4$^+$CD25$^+$ cells (or a portion of those cells) to 1×10^6 cells/ml with RPMI-10 medium.
2. Prepare accessory cells in RPMI-10 as described in Support Protocol 1 or *UNIT 3.5A*. Count cells and adjust accessory cells (or a portion of those cells) to 1×10^6 cells/ml with RPMI-10.
3. Prepare a working solution of 1 μg/ml anti-CD3 in RPMI-10.
4. Prepare a working solution of 200 U/ml IL-2 in RPMI-10.
5. Prepare a working solution of 2 μg/ml anti-CD28 in RPMI-10.

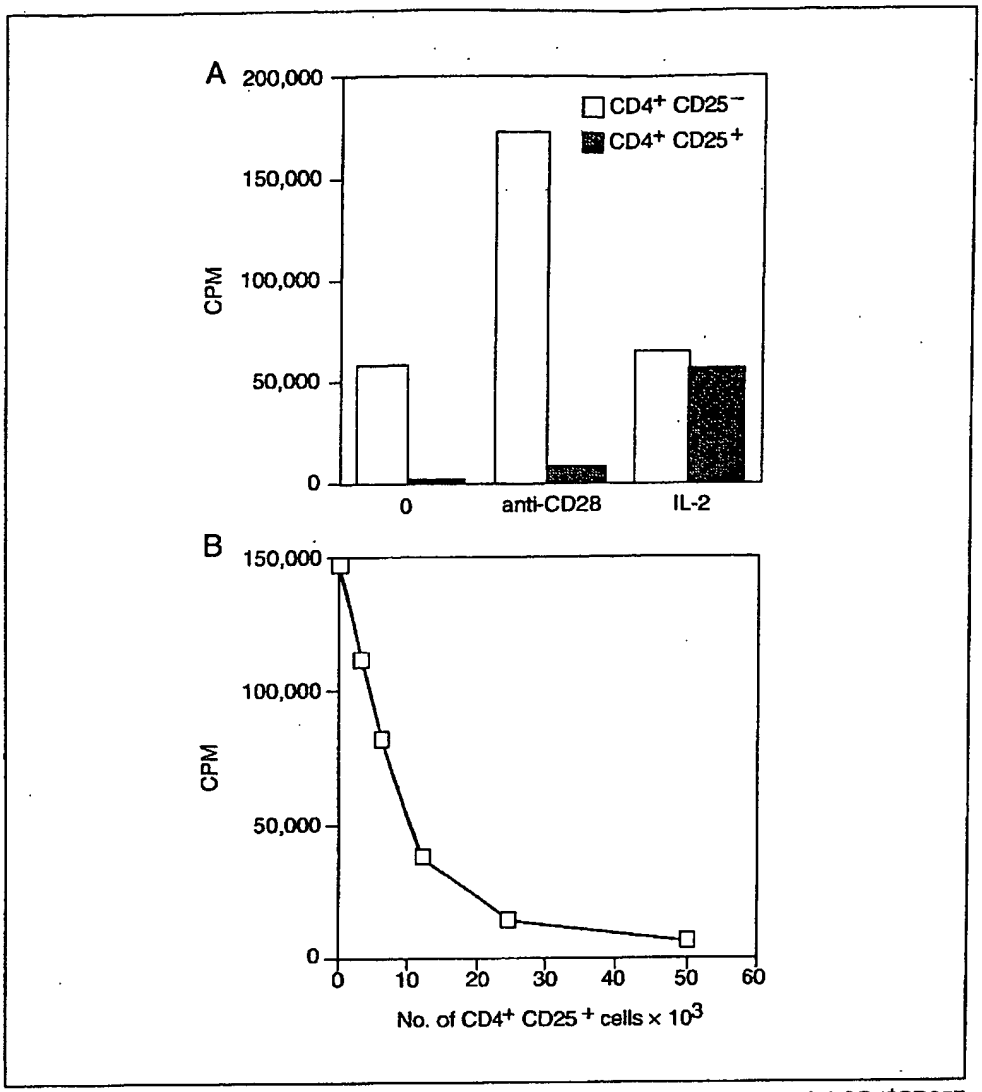

Figure 3.12.1 Analysis of $CD4^+CD25^+$ cell proliferation and suppressor function. (**A**) $CD4^+CD25^-$ (5×10^4) or $CD4^+CD25^+$ (5×10^4) cells were stimulated with AC (5×10^4) and anti-CD3 (0.25 µg/ml) in the absence or presence of anti-CD28 (0.5 µg/ml) or IL-2 (50 U/ml). (**B**) $CD4^+CD25^-$ cells (5×10^4) stimulated with AC (5×10^4) and anti-CD3 (0.25 µg/ml) were co-cultured with the indicated number of $CD4^+CD25^+$ cells. Cultures were incubated for 72 hr and pulsed with 3H-TdR for the last 6 hr of culture.

Prepare to measure the non-responsive state of $CD4^+CD25^+$ T cells

6. Add 50 µl of $CD4^+CD25^-$ cells to each of nine wells of a 96-well flat-bottom microtiter plate and 50 µl of $CD4^+CD25^+$ cells to each of nine wells of a 96-well flat-bottom microtiter plate.
7. Add 50 µl of accessory cells to each of the wells.
8. Add 50 µl of 1 µg/ml anti-CD3 to each of the wells.
9. Add 50 µl of 200 U/ml IL-2 to three wells of the $CD25^-$ cells and three wells of the $CD25^+$ cells.

10. Add 50 µl of 2 µg/ml anti-CD28 to three wells of the $CD25^-$ cells and three wells of the $CD25^+$ cells. To the remaining three wells of each group, add 50 µl of RPMI-10 medium.

These dilutions will result in 5×10^4 $CD25^-$ or $CD25^+$ cells and 5×10^4 T depleted spleen cells per well stimulated with 0.25 µg/ml anti-CD3. The final concentration of IL-2 will be 50 U/ml. The final concentration of anti-CD28 will be 0.5 µg/ml.

Assess suppressive function

11. Add 50 µl of $CD4^+CD25^+$ cells to three wells of a 96-well microtiter plate. Make a series of three to four two-fold dilutions of $CD4^+CD25^+$ cells. Include control wells with 50 µl of RPMI-10 medium only.

A series of two-fold dilutions will result in a starting number of 5×10^4 $CD25^+$ cells per well followed by 2.5×10^4, 1.25×10^4, 0.625×10^4, and 0.3×10^4 cells per well.

12. Add 50 µl of $CD4^+CD25^-$ cells to each well.

13. Add 50 µl of accessory cells to each of the wells.

14. Add 50 µl of 1 µg/ml anti-CD3 to each of the wells.

15. Place microtiter plates in a 37°C, 5% to 7% CO_2 humidified incubator for 3 days (~66 hr).

16. On the morning of the third day, add [^{3}H]thymidine to each well. Return the plates to 37°C, 5% to 7% CO_2 incubator to pulse 6 to 8 hr. Harvest cells using a semiautomated sample harvester and measure cpm in β scintillation counter.

As shown in Figure 3.12.1A, $CD4^+CD25^+$ cells are non-responsive to stimulation with anti-CD3 and APC. The addition of anti-CD28 to $CD4^+CD25^+$ cells stimulated with anti-CD3 and APC does not restore the proliferation of these cells, while the addition of anti-CD28 to $CD4^+CD25^-$ cells significantly enhances their proliferation. However, the addition of IL-2 to $CD4^+CD25^+$ cells results in a proliferative response comparable to that of $CD4^+CD25^-$ cells. In Figure 3.12.1B, the addition of $CD4^+CD25^+$ cells to $CD4^+CD25^-$ cells leads to a dose-dependent decrease in their proliferative response.

THE TWO-STEP SUPRESSION ASSAY: SHORT-TERM ACTIVATION AND EXPANSION OF $CD4^+CD25^+$ T CELLS AND ANALYSIS OF THEIR SUPRESSIVE FUNCTION

ALTERNATE PROTOCOL 3

$CD4^+CD25^+$ T cells represent ~10% of $CD4^+$ T cells. Thus, the purification of $CD4^+CD25^+$ T cells may not yield a sufficient number of cells for some experiments. In order to obtain large numbers of $CD4^+CD25^+$ cells, the cells can be expanded in vitro by stimulating them with platebound anti-CD3 in the presence of IL-2.

After the initial activation for 3 days, the cells are expanded for an additional 3 to 4 days. The suppressive function of the pre-activated $CD4^+CD25^+$ T cells can be tested in a second step as described in Basic Protocol 3, steps 11 to 16. However, under those conditions, the pre-activated $CD4^+CD25^+$ cells are reactivated due to the presence of anti-CD3. One unique property of activated $CD4^+CD25^+$ T cells is that they do not require reactivation through their TCR. After the first step of activation and expansion of $CD4^+CD25^+$ cells, the suppressive function can be assayed in a second step by co-culturing activated $CD4^+CD25^+$ cells with TCR Tg $CD4^+$ T cells activated with peptide. In this manner, the pre-activated $CD4^+CD25^+$ cells are not reactivated.

Additional Materials (also see Basic Protocol 3)

PBS (*APPENDIX 2A*)
Purified $CD4^+$ T cells from a TCR transgenic mouse
Peptide corresponding to the TCR transgenic
24-well flat bottom plates with lids

Prepare cells

1. Purify $CD4^+CD25^+$ T cells in complete RPMI-10 medium supplemented with 100 U/ml IL-2 as described in *UNIT 3.5A*. Count cells and adjust $CD4^+CD25^+$ cells to 1×10^6 cells/ml with RPMI-10/IL-2.

   Unlike conventional T cells, which produce the IL-2 required for their proliferation, $CD4^+CD25^+$ cells do not produce IL-2. Thus, the presence of exogenous IL-2 in the expansion of $CD4^+CD25^+$ cells is critical for their survival.

2. Prepare a working solution of 5 µg/ml anti-CD3 in PBS. Add 300 µl antibody solution to each well of a 24-well plate. Estimate the number of wells to be coated based on the anticipated yield of $CD4^+CD25^+$ cells. Incubate plate 90 min in a 37°C, 5% to 7% CO_2 humidified incubator.
3. Remove the antibody from the plates and wash wells two times with PBS to remove excess antibody.
4. To the wells of the washed plate, add 1 ml (1×10^6) of $CD4^+CD25^+$ cells.
5. Place plates in a 37°C, 5% to 7% CO_2 humidified incubator for 3 days.

   After 3 days, the cells are fully activated, have acquired suppressive function, and can be used as desired. However, they will not have expanded greatly. To expand cell numbers, the cells should be split as follows.

6. After 3 days, split cells 1:3 or 1:4 in RPMI-10 medium supplemented with 100 U/ml IL-2 and return to the 37°C, 5% to 7% CO_2 humidified incubator.

   The cells should be used 3 to 4 days later. Otherwise, they should be split and fed with RPMI-10/IL-2 every 3 to 4 days.

Assess suppressive function

7. Harvest the activated $CD4^+CD25^+$ cells by pipetting up and down rigorously. Centrifuge cells 10 min at 200× *g* (Sorvall H-1000B rotor at ~1000 rpm), 4°C. Wash cells two times to completely remove any remaining IL-2 and resuspend in RPMI-10. Adjust cells to 1×10^6 cells/ml with RPMI-10.
8. Carry out Basic Protocol 3, steps 11 to 16, to measure suppressor function of $CD4^+CD25^+$ cells.

   Under these conditions activated $CD4^+CD25^+$ T cells are reactivated due to the presence of anti-CD3.

9. Prepare a $CD4^+$ T cell suspension in RPMI-10 from TCR transgenic mice as described in *UNIT 3.5A*. Count cells and adjust $CD4^+$ cells (or a portion of those cells) to 1×10^6 cells/ml with RPMI-10.

   The choice of the TCR Tg mouse is up to the investigator and will depend on availability. The amount of antigen (protein or peptide) should be titered prior to experiment and the dose that results in 60,000 to 120,000 cpm chosen.

10. Prepare accessory cells in RPMI-10 as described in Support Protocol 1 or *UNIT 3.5A*. Count cells and adjust accessory cells (or a portion of those cells) to 1×10^6 cells/ml with RPMI-10.
11. Prepare antigen at 4× the desired final concentration with RPMI-10.

12. Add 50 µl of $CD4^+CD25^+$ cells to three wells of a 96-well microtiter plate. Make a series of three to four two-fold dilutions of $CD4^+CD25^+$ cells. Include control wells with 50 µl of RPMI-10 only.

A series of two-fold dilutions will result in a starting number of 5×10^4 $CD25^+$ cells per well followed by 2.5×10^4, 1.25×10^4, 0.625×10^4, and 0.3×10^4 cells per well. Pre-activated cells are more potent suppressors than freshly explanted $CD4^+CD25^+$ T cells. Significant suppression may be seen at lower numbers of added $CD4^+CD25^+$ cells.

13. Add 50 µl of TCR Tg $CD4^+$ cells to each well.

14. Add 50 µl of accessory cells to each of the wells.

15. Add 50 µl of antigen to each of the wells.

16. Proceed as in Basic Protocol 3, steps 15 and 16.

REAGENTS AND SOLUTIONS

Use deionized water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 5.

Percoll solution

Diluent:
45 ml 10× PBS, pH 7.4 (*APPENDIX 2A*)
3 ml 0.6 M HCl
132 ml H_2O
Filter sterilize

70% Percoll solution:
63 ml Percoll (Pharmacia LKB #170891-01)
37 ml sterile diluent (above)
Final osmolarity should be 310 to 320 osM

COMMENTARY

Background Information

Proliferative assays for measuring T cell function have certain advantages and disadvantages compared to the cytotoxic T lymphocyte (CTL) assay described in *UNIT 3.11* or the lymphokine production assays in *UNITS 3.15 & 6.3*. Advantages are that proliferative assays are less time-consuming, less labor-intensive, less cell-consuming, and less expensive than "true" effector T cell function assays. A disadvantage is that antigen specificity is not as easily demonstrated in proliferative assays as in CTL assays, unless antigen-specific clones of proliferating cells are used. Furthermore, the proliferative assay only detects dividing cells instead of measuring true effector T cell function.

It is not clear which T cell function is measured in proliferative assays; the proliferative response should therefore be used solely as general indicators of T cell reactivity. Data obtained in proliferative assays might variously reflect proliferation of CTL, lymphokine-producing T cells, or nonactivated "bystander" cells, and will be severely affected by the function of non–T cells such as accessory cells (see below). Since the majority of T cells respond to and produce IL-2 upon activation, differences in responsiveness in a proliferative assay in part reflect differences in IL-2 production by the responding T cells. Proliferative assays therefore become more meaningful when combined with the lymphokine detection assays presented in *UNITS 3.15 & 6.3*. Since responsiveness to IL-2 is also determined by the levels and functionality of IL-2 receptors, further information will be added by including measurements of IL-2 receptors (*UNIT 6.1*) or by flow cytometry (*UNIT 5.4*). Yet, as a first approximation of cellular activation, proliferative assays are valuable.

The role of immunoregulatory or suppressor T cells has been well documented in the immu-

nologic literature over the past 25 years. Most recently, data on the importance of suppressor cells has been derived from their involvement in responses to infectious agents and tumors, as well as their involvement in mediating transplantation tolerance and in preventing the induction of autoimmune disease (Shevach, 2003). The induction of autoimmune disease by thymectomy at day 3 of life in mice has been considered for many years to be secondary to a deficiency of T suppressor cells that normally develop after day 3 of life. The suppressor cells in this model belong to the minor subpopulation of $CD4^+$ T cells that co-express CD25 and are capable of suppressing the induction of autoimmunity post-3dTx, the induction of autoimmunity induced by transfer of $CD4^+CD25^-$ T cells to *nu/nu* recipients, and the capacity of activated autoantigen-specific T cell clones to induce disease in *nu/nu* recipients.

In an in vitro model system in which the function of these potent $CD4^+CD25^+$ T cells was analyzed, the $CD4^+CD25^+$ cells were themselves completely non-responsive to stimulation by TCR-derived signals in the presence or absence of co-stimulation (Thornton and Shevach, 1998). More importantly, they suppressed the responses of $CD4^+CD25^-$ cells in co-culture studies. Although suppression in many in vivo and in vitro model systems is mediated by the secretion of one of the suppressor cytokines (IL-4, IL-10, or TGFβ), the mechanism of suppression by $CD4^+CD25^+$ cells appears to be mediated by a contact-dependent mechanism that leads to the inhibition of IL-2 gene transcription in the responder T cell population. Thus, the measurement of IL-2 mRNA by quantitative PCR is also a useful assay for the assessment of $CD4^+CD25^+$ T cell function.

It has also been demonstrated that $CD4^+CD25^+$ T cells suppress $CD8^+$ T cell proliferation induced by polyclonal or Ag-specific stimuli (Piccirillo and Shevach, 2001). Most of the effects of regulatory $CD4^+CD25^+$ T cells on $CD8^+$ responders were similar to those seen with $CD4^+$ responders. However, several important differences should be noted. First, in addition to T cell proliferation, the capacity of fresh $CD8^+$ T cells to manifest effector function, such as the production of IFN-γ, was also suppressed. Second, whereas $CD4^+CD25^+$ T cells inhibit the activation of $CD4^+$ responders by primarily blocking IL-2 production, $CD4^+CD25^+$ T cells regulate $CD8^+$ T cell responses both by blocking IL-2 production as well as by lowering responsiveness to exogenous IL-2 and thereby potentially disrupting CD4 help for $CD8^+$ T cells. Finally, $CD4^+CD25^+$ T cells can inhibit T cell activation by directly acting on responder $CD8^+$ T cells in the absence of APC. Although $CD4^+CD25^+$ cells most likely inhibit $CD4^+$ T cells via a T-T cell interaction, the use of peptide-MHC tetramers to stimulate $CD8^+$ responders in a two-cell suppressor assay system directly demonstrated that $CD4^+CD25^+$ cells can mediate their suppressor function via a T-T cell interaction in the absence of APC.

Critical Parameters and Troubleshooting

Parameters affecting the magnitude of T cell proliferative responses include cell concentration, type of medium, source of serum, incubator conditions (CO_2 level and humidity), type and concentration of activating agent, type of responding T cells, type of accessory/stimulator cells, mouse strain, and culture time. Optimal conditions for individual laboratories and experiments must be derived empirically with respect to these variables, but general guidelines are provided below.

A number of agents can be employed in Basic Protocol 1 to induce T cell proliferation (Table 3.12.1). T cells may be activated by pharmacologic means by producing an elevation of intracellular free calcium with a calcium ionophore combined with activation of protein kinase C with a phorbol ester. The most direct means of inducing T cell activation involves stimulation with monoclonal antibodies that interact with the CD3/TCR complex—i.e., anti-CD3, anti-TCR-αβ or -γδ, as well as anti-Vβ antibodies that are capable of interacting with a subset of cells bearing a specific TCR. A vigorous T cell proliferative response of defined subsets can also be induced with certain bacterial toxins known as staphylococcal enterotoxins. These toxins are often referred to as "superantigens" (Marrack and Kappler, 1989) because they stimulate T cells via the variable (*V*) gene segment of the TCR. Different toxins have affinities for different *V*β chains and these specificities make them valuable reagents for activating T cells. The activating capacity of toxins is also dependent on their ability to bind to MHC class II molecules (i.e., responding T cells react with the toxin/class II complex); thus, responsiveness varies with the mouse strain used. Lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) have been widely used for many years to activate T cells. Although the precise mechanism of ac-

tion of these agents is unknown, it is likely that lectins activate T cells by indirectly cross-linking the TCR because TCR-negative cells will not respond to these agents. Lastly, it is also possible to induce T cell activation with monoclonal antibodies to cell-surface antigens other than the TCR; this protocol employs the G7 monoclonal antibody, one of the most effective of the anti-Thy-1 activators (Gunter et al., 1984).

When comparing the reactivity of different cell populations, it is essential to perform dose-response assays for responder T cells and activating agents and for both responder and stimulator T cells (in MLR), since each population may yield optimal responses at different cell numbers. This may reflect differences in frequency of responding cells, and hence may indicate a need to perform limiting dilution assays (*UNIT 3.15*). Since peak responsiveness of different populations of T cells may occur at different times, it is also essential to perform kinetic experiments—i.e., compare responsiveness at days 2, 3, 4, and 5.

Differences in responsiveness need not necessarily be due to differences in the frequency of responding T cells, but may also indicate differences in the efficacy with which co-stimulatory activity or "second signals" are delivered by the accessory cells present in different cell populations. The type of interactions pertinent to the generation of primary responses by T cells is explained in the commentaries of *UNITS 3.8 & 3.11 & 3.13*. Specific requirements for inducing activation with immobilized antibodies have been described (Staerz and Bevan, 1986; Hathcock et al., 1989; Jenkins et al., 1990). A responding cell population completely devoid of accessory cells (such as purified populations of splenic or lymph node T cells or cloned T cells) will yield fine responsiveness in an MLC, since accessory cell function is provided by the stimulator cells; however, the same population will generally not yield responses when mitogens, antigens, or enterotoxins are used. In such a setting, accessory cells may also function as antigen-presenting cells (APC). Addition of irradiated or mitomycin C–treated syngeneic sources of accessory cells (either whole spleen cells or purified APC; see Support Protocol 1) can be used to restore responsiveness in purified T cells. The need for accessory cells can sometimes be bypassed when anti-TCR monoclonal antibodies are coupled to plastic, or when certain anti-Thy-1 monoclonal antibodies are used; however, these conditions do not necessarily result in optimal responsiveness (Jenkins et al., 1990).

The level of [^{3}H]thymidine incorporation should not be regarded only as a reflection of cellular proliferation: some nondividing cells will synthesize DNA and "cold" thymidine released by disintegrating cells will compete with incorporation of labeled thymidine. Therefore, measurements of DNA synthesis should be accompanied by counting viable cells over the length of the culture period if a true estimate of cellular proliferation is to be obtained. Of course, cell death of nonactivated cells will also interfere with the accuracy of this last parameter.

The sensitivity of proliferation assays is such that small errors in cell numbers will result in large differences in [^{3}H]thymidine incorporation values. When values obtained in triplicate cultures correspond poorly (e.g., >5% difference in cpm values >1000), technical problems such as cell clumping, dilution, and pipetting should be considered. Excessively high values may be obtained from contaminated wells, as [^{3}H]thymidine will be incorporated into replicating bacteria; therefore, it is good practice to check the wells from microtiter plates under an inverted microscope for contamination. Contamination may also interfere with proliferation of the activated lymphocytes.

It is also useful to check for blast formation by microscopic examination of the cultures: activated lymphocytes will tend to enlarge, and detection of blasts will give a general indication of successful activation.

The main problem that may occur with proliferative response assays is high levels of background [^{3}H]thymidine incorporation in control cultures without antigens. This problem is frequently due to the fetal bovine serum (FBS) used to supplement the cultures, which may be mitogenic for B cells. Different lots of FBS should be screened to select those that are nonstimulatory or only weakly stimulatory in the absence of other stimuli, and that support strong proliferative responses upon antigenic stimulation of T cells.

If flat-bottom microtiter plates are used in the procedure and weak responses occur, it may be useful to switch to round-bottom plates. The authors' laboratory has found consistently better responses in round-bottom plates when thymocytes are used as responder cells or with slight alloantigenic differences between responding and stimulating cells. In addition, antibody-mediated experiments yield better results with

round-bottom plates. Presumably, this reflects better cell contact obtained in such plates; optimal responses will almost certainly occur at different cell numbers than in flat-bottom plates and densities will have to be adjusted accordingly.

Although satisfactory responses to most alloantigens can be obtained with complete RPMI-10 medium, it may be necessary to compare different media. This need arises when the proliferative responses are weak (i.e., when [^{3}H]thymidine values for activated cultures are <10-fold higher than those for control cultures) and may occur under various circumstances: weak alloantigenic differences between responder and stimulator cells, weak T cell proliferative function in the responder cells or diminished APC function in the stimulator cells due to experimental manipulations, or a low precursor frequency of responding T cells. Thymocytes in particular do not contain a high level of responding T cells. Frequently, proliferation can be improved when complete Clicks or Dulbeccos media are used (with additives as described in *APPENDIX 2A*), presumably because these media contain additional nutrients and have an osmolarity more compatible with mouse serum than RPMI.

When RPMI is used as medium, 5% CO_2 will be sufficient, but for other media, a 7.5% CO_2 concentration in the incubator will be more satisfactory. Generally, the buffering capacity of DMEM is insufficient at 5%, but fine at 7.5%. Much will also depend on the proliferative activity of the responding population of T cells (e.g., vigorous proliferation will reduce the pH in the cultures); it is therefore recommended to compare responsiveness in initial pilot experiments in incubators set at different CO_2 concentrations.

The culture period required for stimulation—after which the cells are to be labeled—varies for different laboratories, media, and types of responding and stimulator cells. Conditions eliciting weak responses, such as those obtained with thymocytes or a weak alloantigenic difference, will require a longer culture period (5 to 6 days) than those which elicit a higher frequency of responding T cells (3 to 4 days). Because laboratory conditions vary, it will be necessary to run a kinetic assay to determine the optimal time for T cell proliferation. Addition of [^{3}H] thymidine on days 2, 3, 4, 5, and 6 will provide a useful test; further extension of the culture period will not yield any improvements, due to exhaustion of nutrients in the medium.

Suppression in vitro requires that the CD4$^+$CD25$^+$ T cells be activated via the TCR since the antigen-specific response of naive T cells from TCR transgenic mice is not suppressed by wild-type CD4$^+$CD25$^+$ cells, while the responses of the same cell mixture to anti-CD3 stimulation are completely suppressed. In the assessment of CD4$^+$CD25$^+$ T cell function, it may be necessary to titer the amount of anti-CD3 used to stimulate the CD4$^+$CD25$^-$ if significantly lower or higher counts than expected are obtained. In the case of significantly lower counts from the CD4$^+$CD25$^-$ cells, it is difficult to obtain credible suppression. In the case of high counts, it is more difficult for CD4$^+$CD25$^+$ cells to suppress a vigorous proliferative response. Although the responses of mouse CD4$^+$CD25$^-$ cells to soluble anti-CD3 in the presence of normal T-depleted spleen cells are easily suppressed, the responses to plate-bound anti-CD3 are not suppressed. However, human CD4$^+$CD25$^+$ cells are able to inhibit the response of human CD4$^+$CD25$^-$ T cells to plate bound anti-CD3 (Baecher-Allan et al., 2001). In addition to polyclonal stimuli such as anti-CD3, stimulation with allogeneic cells is frequently employed when using both mouse and human cells (Cohen et al., 2002).

CD4$^+$CD25$^+$ T cells can be expanded quite efficiently when activated via their TCR in the presence of IL-2 and remain anergic and suppressive (Thornton and Shevach, 2000). More importantly, in contrast to freshly isolated CD4$^+$CD25$^+$ cells, activated CD4$^+$CD25$^+$ cells are powerful suppressors of the responses of CD4$^+$CD25$^-$ transgenic T cells to a variety of antigens. When co-cultured with antigen-stimulated TCR Tg CD4$^+$ cells, the CD4$^+$CD25$^+$ do not require restimulation through their TCR. However, when restimulated with anti-CD3 in a second culture, their capacity to suppress anti-CD3 stimulation of CD4$^+$CD25$^-$ T cells is enhanced four- to six-fold when compared to freshly isolated CD4$^+$CD25$^+$ cells.

As CD4$^+$CD25$^+$ T cells require IL-2 during the pre-activation step, care must be taken to use very pure populations of CD4$^+$CD25$^+$ cells. The elimination of CD8$^+$ cells as described in *UNIT 3.5A* is critical. CD8$^+$ T cells preferentially expand to IL-2 and contamination of as little as 2% CD8$^+$ cells can increase to 50%. Characterization of the pre-activated CD4$^+$CD25$^+$ cells should include analysis by flow cytometry to assess the purity of the expanded population.

A 6- to 8-hr pulse on day 3 is ideal, but if an overnight pulse is preferred, [^{3}H]thymidine

should be added on day 3 and the plates harvested on day 4, as the stimulation conditions are sub-optimal.

Anticipated Results

For proliferative assays described in Basic Protocol 1, which activate the majority of the responding T cells, responses of 100,000 cpm should be obtained; in the MLR or following activation with monoclonal antibodies to subpopulations of T cells (anti-Vβ), responses up to 100,000 cpm may be observed; however, measurements of 20,000 cpm (with tight standard errors) can be quite satisfactory. Background values of <1000 cpm should be expected. Reported results (as described in step 9a) should be mean cpm of experimental wells minus background cpm (Δ cpm).

From $CD4^+CD25^-$ cells, proliferative responses of 60,000 to 150,000 cpm are desirable, while the addition of IL-2 will slightly increase the response. However, highly purified $CD4^+CD25^+$ cells from normal, healthy mice do not proliferate in response to T depleted spleen and anti-CD3 alone. A slight response of 1000 to 2000 cpm is acceptable. The addition of anti-CD28 may yield a modest response (5000 to 20,000 cpm) but should not be significantly higher. Higher counts may indicate contaminating $CD4^+CD25^-$ cells or effector $CD4^+CD25^+$ cells from unhealthy mice. With the addition of IL-2 though, a proliferative response comparable to that of $CD25^-$ cells should be obtained.

When $CD4^+CD25^+$ cells are co-cultured with $CD4^+CD25^-$ cells, the proliferative response of $CD4^+CD25^-$ should be inhibited in a dose-dependent manner. From 1.25×10^4 $CD4^+CD25^+$ cells, 50% inhibition is expected, while 85% to 98% inhibition should be obtained in the presence of $2.5–5.0 \times 10^4$ $CD4^+CD25^+$ cells.

The expansion of $CD4^+CD25^+$ cells when stimulated with plate-bound anti-CD3 in the presence of IL-2 is generally 10- to 20-fold after 7 days.

Time Considerations

The time required to set up proliferative assays is not more than a day, with the number of hours depending on the number of different groups of responder cells that must be prepared. The time required for incubation of cells ranges from 2 to 6 days, as noted above in Critical Parameters and Troubleshooting. Following an additional 18- to 24-hr incubation period for pulsing, harvesting the cells and measuring cpm will require several hours depending on the number of plates (~15 min for harvesting each plate and ~100 min for counting each plate at 1 min/sample).

Once purified cells are obtained, the time to set up the proliferation assays requires ~30 to 60 min. Somewhat more time may be necessary if additional conditions for testing $CD4^+CD25^+$ cells are desired.

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